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An Examination of an Alternative Use for the Fear-Potentiated Startle Paradigm and the Effects of Angiotensin Type 1 Receptor Inhibition on Extinction Learning

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An Examination of an Alternative Use for the Fear-Potentiated Startle Paradigm and the Effects of Angiotensin Type 1 Receptor Inhibition on Extinction Learning

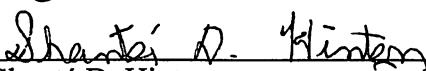
A thesis submitted in partial fulfillment of the requirement
for the degree of Bachelor of Science in Neuroscience from
The College of William and Mary

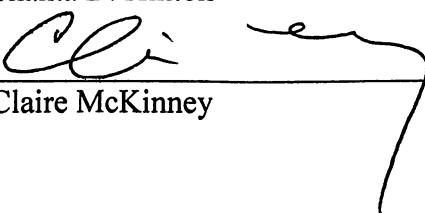
by

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Abstract

Background – PTSD is a chronic and debilitating mental illness with limited available treatments. New medications, including Angiotensin Type 1 Receptor inhibitors, have been found to improve extinction learning retention in rodents and could be a means of improving the efficacy of exposure and cognitive based therapies for PTSD and other anxiety related disorders. Studies of new medications use an ideal model of extinction that does not mirror the inconsistencies of human therapy and recovery. The present experiment attempted to model the inconsistent nature of exposure therapy and examine whether the AT1R inhibitor, losartan, would still improve extinction learning.

Methods – Using the fear-potentiated startle paradigm as an extinction paradigm, the study investigated whether acute treatment with losartan, an angiotensin type 1 receptor inhibitor, could still augment extinction retention. Following Pavlovian fear conditioning, an animal model for PTSD, subjects were treated to losartan and tested for extinction learning over the course of two days.

Results – Groups treated with losartan showed consistent significant fear-potentiated startle in both tests following initial fear conditioning. In contrast, groups treated with saline did not show significant changes in fear-potentiated startle or trend towards a significant increase in startle reactivity on either day.

Conclusions – The significant increase exhibited by the group treated with losartan could be an implication that losartan could augment fear memory as well as extinction retention, but further study will be necessary to confirm whether the Fear-Potentiated Startle paradigm can truly demonstrate fear extinction in animals.

Introduction

Post-traumatic Stress Disorder (PTSD) is an often chronic and debilitating mental illness that impacts thousands of individuals across the country. According to the most recent Diagnostic and Statistical Manual of Mental Health Disorders (DSM V), an individual may develop PTSD after exposure to some type of severe trauma such as “actual or threatened death, serious injury, or sexual violence.” While most individuals will exhibit acute symptoms of distress, such as a negative emotional state, intrusive thoughts or dreams, and hypervigilance, these symptoms commonly resolve over a few weeks (Lancaster, Teeters, Gros, & Back, 2016; DSM V, 2013). For individuals who suffer from PTSD, symptoms take a significantly longer period to resolve. They also may show more severe and debilitating symptoms such as dissociative reactions, prolonged psychological distress or physiological reactions to internal or external cues related to the trauma, and inability to remember key aspects of the traumatic event for a period that exceeds at least one month (DSM V, 2013). For many individuals, this disorder can severely lower quality of life by impacting the ability to concentrate on work and personal interests, take pleasure in previously enjoyed activities, or form meaningful relationships (Gold, Douglas, Thomas, Elliott, Rao, & Miaskowski, 2012; Kearney & Simpson, 2014).

Importantly, PTSD is also one of the more common mental health disorders in the United States with an estimated 8.3% lifetime prevalence in 2013 (Kessler, Petukhova, Sampson, Zaslavsky, & Wittchen, 2012; Kilpatrick, Resnick, Milanak, Miller, Keyes, & Friedman, 2013). As a result, seeking a greater understanding of PTSD and potential treatments for the disorder is vital for individuals who suffer from it.

What are the current treatments for PTSD?

Currently, the best known treatments for PTSD are psychotherapeutic treatments including exposure therapy, cognitive processing therapy, and relaxation-based therapies (Lancaster et al., 2016; Cusack et al., 2016). Unfortunately, some individuals with PTSD do not respond effectively to psychotherapy and further, there is a fairly large proportion of individuals who drop out of psychotherapy (Lancaster et al., 2016; Najavits, 2015). A 2016 review estimated that dropout rates for the most effective forms of psychotherapy range from 10-38% for prolonged exposure therapy and around 20% for cognitive processing therapy (Lancaster et al., 2016).

While pharmacotherapy is available for patients with PTSD, there currently is limited research and understanding of the benefits that some medications can have. Selective serotonin reuptake inhibitors (SSRIs) have been the most heavily studied, being used in the treatment of depression and anxiety disorders as well (Davidson et al., 2003). Evidence has shown that extended treatment with SSRIs (36+ weeks) can reduce risk of PTSD relapse and may also improve response to psychotherapies; however, SSRIs can have significantly delayed impact, or be ineffective for some patients (Lancaster et al., 2016, Davidson et al., 2001).

Instead of targeting specific symptoms of PTSD, some newer research has taken a more multi-faceted approach in developing pharmacotherapies. A common theory is that, at its core, PTSD is largely tied to an inability to effectively extinguish a fear memory resulting in a chronically elevated fear response to stimuli that are associated with a traumatic event (Milad, Orr, Lasko, Chang, Rauch, & Pitman, 2008; Milad, et al., 2009). Noting that psychotherapies have been highly effective, a number of studies have focused on researching treatments that may

assist patients by improving learning retention for therapeutic treatments, or otherwise improve responsiveness to psychotherapeutic treatments (Lancaster et al., 2016).

Medications that can augment extinction learning have recently become particularly popular. For example, d-Cycloserine (DCS), an NMDA receptor agonist, has been found to improve extinction learning in a number of animal studies (Walker, Ressler, Lu, & Davis, 2002). In recent years, human studies have also found treatment with DCS to have a small but significant impact on improving patient responses to psychotherapy (Mataix-Cols, et al., 2017). The results have been mixed, however. Other studies have found that treatment with DCS may sometimes actually worsen symptoms of PTSD, potentially because DCS may augment a trauma memory if an exposure therapy session is unsuccessful (Litz, et al., 2012; Reinecke & Harmer, 2015). In other words, the DCS does not assist the patient in discriminating between safety and threat cues in an extinction session (Reinecke, et al., 2018).

PTSD is a prevalent disorder in the United States, and availability and access to preferred forms of psychotherapy are limited for large portions of the population. A fast-acting drug that could effectively enhance learning of safety cues without exacerbating trauma memory could significantly improve recovery time for individuals who suffer from PTSD. This study examines whether an anti-hypertensive drug, losartan, can augment extinction learning, and seeks to examine whether this drug may still improve extinction learning in mildly stressful environments.

The Renin-Angiotensin System

The renin-angiotensin system (RAS) is the body's primary method of long-term regulation of the cardiovascular system and does so primarily through the production of the peptide hormone, Angiotensin II (Ang II) (Sparks, Crowley, Gurley, Mirotsoy, & Coffman,

2014). Ang II interacts with two primary receptors, the Angiotensin Type 1 receptors (AT1R) and the Angiotensin Type 2 receptors (AT2R), which are located throughout the body with a high concentration in the brain (Unger, Steckelings, & Dzau, 2015; Sparks et al., 2014; DSM V, 2013). These two receptors are both seven-transmembrane G-protein coupled receptors (GPCRs) (Unger et al., 2015). The Ang II interaction with the AT1 receptor causes a signaling cascade that results in vasoconstriction, bodily salt retention, and inflammation among other symptoms related to hypertension (Unger et al., 2015). As a result, drugs such as AT1R specific angiotensin receptor blockers (ARBs), and angiotensin converting enzyme inhibitors (ACE-Is), which block the synthesis of Ang II, are often used to target this pathway to treat patients who suffer from hypertension and other cardiovascular diseases (Marvar, Goodman, Fuchs, Choi, Banerjee, & Ressler, 2014).

Importantly, it recently has been found that ARBs not only treat symptoms of hypertension but can also cause the long-lasting reversal of negative effects, including fibrosis of heart and blood vessels and hypertrophy (Unger et al., 2015). These healing effects have been attributed to two primary factors. First, activation of AT1 receptor signaling cascade can result in the production of AT1R mRNAs that then code for new AT1 receptors (Unger et al., 2015, McKinley et al., 2003). By antagonizing the AT1 receptor, ARBs indirectly inhibit any potential increases in AT1R activity (McKinley et al., 2003). The second theory is that inhibiting Ang II interaction with the AT1 receptor results in an excess of Ang II in the body that can then interact with the AT2 receptor (Unger et al., 2015). The AT2 receptor has been found to counter many effects caused by AT1 receptor activation (Unger et al., 2015). Activation of the AT2 receptor has been found to induce signaling cascades that result in vasodilation, anti-inflammation, and

natriuresis, among other effects including anti-fibrosis and neuroregeneration or protection (Unger et al., 2015).

Neuroprotective Effects of Angiotensin Receptor Blockers

AT1 receptors are located in multiple areas of the brain, but importantly are located in parts of the limbic system, including the amygdala, and the bed nucleus of the stria terminalis (BNST) (McKinley et al., 2003). AT1 receptors are also highly localized in the hypothalamus and influence the hypothalamic pituitary adrenal (HPA) axis which could link the receptor's association with fear memory (Marvar et al., 2014; McKinley et al., 2003). The AT2 receptor is localized to several areas that do not overlap with the AT1 receptor including the thalamus and cerebellum, though in humans, AT1 and AT2 receptors are both localized in the cerebellar cortex (Namsolleck, Culman, & Unger, 2015). The primary functions of the cerebellar cortex include sensorimotor and vestibular control, it may also play a role in cognition (Schmahmann, 2018)

Recent studies have shown that RAS may also play a role in the regulation of stress-related disorders and neuropathologies. Activation of the AT1 receptor results in inflammation in the brain that has been associated with stroke, ischemia, and hypertension (Saavedra, 2012; Saavedra, 2004). Additional resulting neuronal damage has been associated with reduced cognition, depression, and anxiety (Saavedra, 2012; Saavedra, 2004). Other studies have found that acute stress can cause a surge of AT1R mRNA in the paraventricular nucleus of the hypothalamus which may result in increased levels of AT1 receptors in the brain thus elevating stress responses over time (Dumont, Rafrafi, Laforest, & Drolet, 1999).

ARBs appear to mediate these effects. ARBs are selective antagonists for AT1 receptors and compete with Ang II for the AT1R binding site. As noted previously, ARBs not only reduce symptoms caused by activation of the AT1 receptor, but also result in the reversal of symptoms

including pathological neuronal remodeling and cerebrovascular inflammation as a result of increased activation of the AT2R (Saavedra, 2004; Unger et al., 2015; Bregonzio, Seltzer, Armando, Pavel, & Saavedra, 2008).

Pre-treatment of animals with the ARB, candesartan, prevented a neuroendocrine response to isolation and the development of stress-related ulcers in mice (Saavedra, 2004). In a more recent study, a knockdown of the AT1 receptor in the subfornical organ (SFO) of the brain in mice was found to significantly decrease Ang II binding to the AT1 receptor, and by proxy reduce the production of corticotrophin, a stress hormone associated with the AT1R signaling cascade (Krause et al., 2011).

Treatment with ARBs has been further demonstrated to improve mood and ameliorate the progression of Alzheimer's disease (Saavedra, 2004; Gard, 2004). ARBs from the sartan family (i.e., losartan, valsartan, and candesartan) have been found to reduce stress levels by reducing corticosterone release (Armando, et al., 2001). They also have been found to relieve depressive symptoms by acting as indirect agonists for the AT2 receptor (Diniz, Casarotto, Fred, Biojone, Castrén, & Joca, 2018).

The association of the renin-angiotensin system with symptoms of stress has been conducive to examinations of the impacts that ARBs may have on reducing symptoms in individuals with PTSD. In 2012, a longitudinal study of 500 hypertensive patients who had also suffered from severe trauma was conducted (Khoury, Marvar, Gillespie, Wingo, Schwartz, & Bradley, 2012). The patients were receiving a variety of treatments for hypertension, including diuretics, beta-blockers, calcium channel blockers, angiotensin converting enzyme inhibitors (ACE-I) and ARBs. ACE-Is prevent the production of Ang II, thus indirectly reducing activation of the AT1R (Khoury et al., 2012). The study found that a select group of individuals prescribed

ACE-Is or ARBs showed reduced symptoms of hyperarousal or intrusive thoughts characteristic in PTSD (Khoury et al., 2012). In contrast, patients taking other anti-hypertension medications that do not interact with the RAS showed no significant decrease in PTSD symptoms (Khoury et al., 2012). This suggested that the reduction of PTSD symptoms was associated with RAS neuroregulation (Khoury et al., 2012).

In an effort to better understand the association of the RAS with PTSD symptoms, various animal studies have been conducted to examine the mechanism responsible for its anxiolytic effects. It is known that ARBs interact with AT1 receptors both within the circumventricular organs of the brain that are unprotected by the blood-brain barrier, as well as with AT1 receptors in parts of the brain that are protected by the blood-brain barrier including the amygdala and hypothalamus (Saavedra, 2004; McKinley, et al., 2003).

The study that influenced this thesis was conducted by Marvar et al. (2014) and sought to examine whether the ARB, losartan, might impact fear memory or have an impact on learning. Marvar et al.'s study utilized a traditional Pavlovian fear conditioning model that has been used to model PTSD in animals. This model is used for the ease it provides in assessing fear acquisition, expression, inhibition, and extinction learning. To summarize the results of the study, treatment with losartan was found to have no impact on the acquisition of fear (Marvar et al., 2014). Further, the animals in the study showed no enhanced fear extinction following acute administration of losartan fear (Marvar et al., 2014). In an extinction training session 24 hours following initial treatment, however, the animals treated with losartan did show enhanced retention of fear extinction (Marvar et al., 2014). Given losartan is an anti-hypertensive drug, it is important to note that reduced fear expression was not a result of acutely lowered blood pressure.

The 10 mg/kg dosage of losartan appeared to have no impact on baseline blood pressure (Marvar et al., 2014).

From these data, it does not appear that losartan impacts fear acquisition or recall of fear memory. Instead, these results imply that losartan may improve retention of extinction learning (Marvar et al., 2014). Similar to d-Cycloserine, this information implies that losartan could potentially be useful as a therapy enhancing drug (Reinecke, et al., 2018; Marvar et al., 2014). Assisting patients with PTSD with the extinction of aversive memories could improve the efficacy and efficiency of exposure therapy.

As noted, however, d-Cycloserine has been associated with some adverse effects and may worsen a fearful memory by enhancing reconsolidation following an unsuccessful therapy session or stressful event (Reinecke, et al., 2018; Reinecke & Harmer, 2015). That said, d-Cycloserine acts on the glutamatergic system while ARBs interact with the RAS (Tomek, Nemirovsky, Olive, & LaCrosse, 2013; Marvar et al., 2014).

By augmenting the healing effects of AT2 receptors and inhibiting the inflammatory effects of the AT1 receptor, the ARBs may influence extinction learning differently than d-Cycloserine (Reinecke, et al., 2018; Unger et al., 2015). There remains a question as to whether losartan may allow individuals to discriminate between safety and threat cues while augmenting consolidation of fear extinction (Reinecke, et al., 2018). In some ways, the effects of ARBs have already been observed in the Khoury (2012) longitudinal study when the helpful effects of ARBs were minimized by the combination of the treated group with participants who were treated with ACE-Is and further by the small number of participants diagnosed with PTSD. The purpose of this study is to further examine the impact of losartan on extinction learning, utilizing another fear extinction paradigm.

Fear-Potentiated Startle

In a classic fear conditioning procedure, a subject receives an aversive, unconditioned stimulus (US) such as a shock. This US is immediately preceded by a “conditioned stimulus” (CS) such as a bell or light that the subject comes to associate with the unconditioned fear stimulus. When the subject is re-exposed to the CS, they will exhibit a fear response even in the absence of the US. An extinction paradigm mimics human exposure therapy by repeatedly exposing an animal to a feared CS in a safe environment and without the US until the animal no longer exhibits fearful behavior to the CS.

As follow-up research for Marvar (2014), this study examined fear extinction in mice using the fear-potentiated startle paradigm. The fear-potentiated startle (FPS) paradigm presents an aversive but non-harmful stimulus (i.e., a white noise sound) following a conditioned stimulus (i.e., a tone). This procedure is often used to assess whether fear conditioning has been successful since potentiated startle only occurs following Pavlovian classical conditioning or whether a fear response has been effectively extinguished (Davis, Falls, Campeau, & Kim, 1993; Walker et al., 2002). The FPS pathway has been useful in previous studies, because it allows researchers to infer a state of fear from a heightened reactivity and unlike freezing behavior, which is also often measured to assess a state of learned fear in mice and rats, the startle response and its neural mechanism is conserved between animals and humans (Davis, 2006).

Some studies have shown that in humans, fear-potentiated startle was higher in individuals who suffer from PTSD or other anxiety related disorders as opposed to participants without anxiety or trauma related disorders when the participants sensed that they were in a threatening environment (Morgan III, Grillon, Southwick, Davis, & Charney, 1995; Melzig, Weike, Zimmermann, & Hamm, 2007; Dunning & Hajcak, 2015; Norrholm, et al., 2011). From

a macro perspective, many individuals suffering from PTSD may be unable to leave the environments in which they have experienced trauma or may have experienced trauma that is easily triggered by commonplace events (Birrer, Michael, & Munsch, 2007; Priebe, Kleindienst, Zimmer, Koudela, Ebner-Priemer, & Bohus, 2013; Kilpatrick et al., 2013). As a result, these individuals may frequently find themselves in environments of heightened or potentiated stress.

Though it has not been used as one previously, the paradigm is described as an extinction procedure since the conditioned stimulus is presented without the initial unconditioned stimulus (i.e., a footshock). That said, previous research implies that using the FPS paradigm as an extinction procedure alone may result in delayed extinction to a conditioned stimulus in comparison to regular extinction training (Walker et al., 2002). This delay may be caused by the startle stimulus creating a disruption to CS processing or an increase in fear in relation to the CS (Walker et al., 2002). Extending this information to the human experience, humans with PTSD may frequently find themselves in situations of heightened stress that may coincide with an aversive cue as opposed to a safety cue. Thus, the FPS paradigm may be a way to model heightened stress experienced by humans in animal studies.

In many ways, this study mirrors the Marvar (2014) study that hypothesized that animals treated with losartan have an enhanced consolidation of memory. This study sought to examine whether using an FPS paradigm would result in delayed extinction learning as a result of coupling the conditioned stimulus with the non-harmful, but still aversive startle stimulus. This research further sought to understand whether fear conditioned animals who were treated with an acute dose of losartan and exposed to the FPS paradigm would have an increased retention of extinction learning in the second day of treatment in comparison to an untreated group. While the FPS paradigm presents a slightly aversive stimulus, it was hypothesized that this would not

inhibit the organism's ability to extinguish a fear response to the conditioned stimulus since losartan ameliorates the AT₁ receptor induced stress response.

The study has been divided into two experiments. The first experiment provided preliminary evidence necessary to implement the second experiment. Though the experiments are largely similar, there are some significant differences in the methods and length of each process.

Experiment 1

Method

Subjects

All mice (N = 8) in the experiment were 3-4 month old wild type C57BL/6J males from Jackson Laboratory (Bar Harbor, ME). All procedures were approved by the Institutional Animal Care and Use Committee of George Washington University and were in compliance with the National Institutes of Health guidelines.

Drugs

Losartan (Sigma-Aldrich, catalog no. 61188), a selective AT₁ receptor antagonist, was administered intraperitoneally (i.p.) at a dose of 10 mg/kg in 0.2 ml of a vehicle of 0.9% isotonic sterile saline. Saline was administered during control sessions. The mice received a single dose of losartan or saline 40 minutes prior to Fear-Potentiated Startle test 1.

Habituation and Fear-Potentiated Startle Pretest

Over the first three days, the mice were exposed and acclimated to a non-restrictive clear acrylic cylinder (SR-Lab startle response system, San Diego Instruments) attached to an accelerometer that measured mV reactivity. On the first day and second day, subjects were exposed to a 17 min baseline reactivity test which consisted of 48 0.5 sec white noise bursts at varying decibel levels:

65, 81, 90, 95, 100, 105, 110, and 115 dB and a randomized inter-trial interval (between 0-30 sec). This information was used to select a dB level (100 dB) that evoked a recognizable startle response without causing a ceiling effect.

On the second day, the animals received a pre-FPS test to measure baseline potentiation to the tone conditioning since fear reactivity in mice varies greatly within groups (Falls, 2002). The FPS test consisted of 5 min silence and 10 “leader trials” (0.5 sec 100 dB noise burst) to acclimate the mice to the chamber and stabilize reactivity. This was followed by 15 tone (30 sec, 6 kHz, 60 dB) trials that co-terminated with a white noise burst (0.5 sec, 100 dB) and were interspersed with 15 noise alone trials (0.5 sec, 100 dB), and 15 no-stimulation trials (30 sec silence). No-stimulation trials were used to measure baseline movement within the cylinders. Each trial was followed by a 30 sec inter-trial interval.

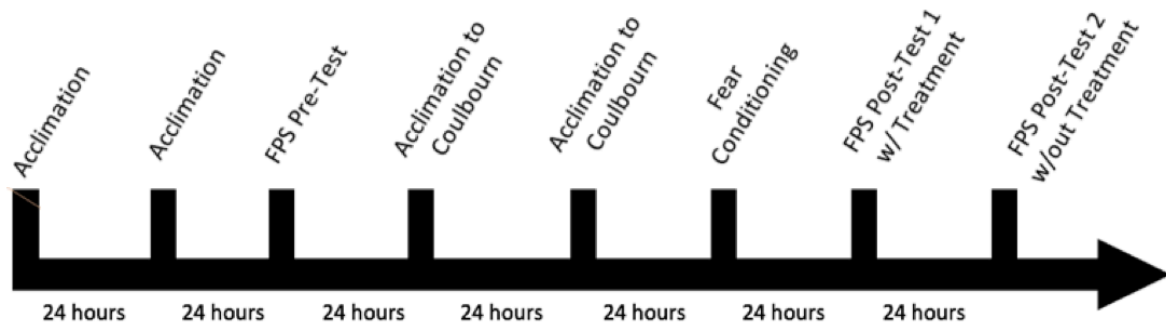
Cue Fear Conditioning

Auditory fear conditioning was performed in conditioning test cages (7" × 7"D12"H; model H10-11M-TC) equipped with overhead cameras and grid shock floors (H10-11M-TC-SF). Test cages were enclosed in sound attenuating isolation cubicles (Model H10-24T; Coulbourn Instruments, Holliston, MA, United States). Animals were habituated to the chambers in two 20 min sessions two days prior to fear conditioning protocol and both the saline and losartan groups received to the fear conditioning protocol. The protocol consisted of 5 min habituation period followed by 5 CS-US pairings: a 30 sec auditory tone (6 kHz, 75 dB) co-terminating with a mild footshock (0.5 sec, 0.5 mA). There was a 3 min 30 sec inter-trial interval between each pairing. The conditioning chambers were cleaned with 70% ethanol between each trial and the animals were returned to their home cages for 24 hours following conditioning.

Fear-Potentiated Startle Extinction Testing

The FPS extinction test was performed using the SR-Lab Startle Response System (San Diego Instruments). Animals were placed in non-restrictive acrylic cylinders attached to an accelerometer that measured mV reactivity. The FPS protocol used in the pretest was also used in both posttests. Mice were injected with losartan or saline 40 min prior to the first FPS test and returned to their home cages. The conditioning chambers were cleaned with 70% ethanol between each test and the animals were returned to their home cages for 24 hours following FPS extinction training.

Timeline of Behavioral Assessment for Experiment 1



Analysis

The conditioning test cages come equipped with a program to calculate %Freezing in animals. Fear conditioning was assessed using data for %Freezing during each 30 sec tone. Fear conditioning was then calculated by comparing freezing behavior prior to the initial shock (Pre-US freezing) to freezing behavior following the initial shock (Post-US freezing). A two-way ANOVA with time and drug as factors was used to confirm conditioning and similarity between treatment groups.

Fear-potentiated startle was calculated based on a ratio comparing tone + noise trials to noise alone trials using the following formula: Percent fear-potentiated startle (%FPS) = [(tone + noise burst – noise burst alone)/noise burst alone] × 100. To determine fear potentiation, the average

reactivity in mV was calculated for each animal for each set of trials: [No-stimulation], [noise burst alone], and [tone + noise]. Percent FPS was then calculated using the above formula for each animal.

Fear Conditioning was assumed based on a significant increase in FPS from Pretest to Posttest 1 (Falls, 2002). It was assumed that animals returned to baseline and had extinguished to the fear cue if there was no significant difference between Pretest and Posttest 2. This assumption was based off the data that showed that animals who had received extinction treatment in other experiments no longer exhibited FPS when exposed to the CS prior to a startle stimulus. (Walker, Ressler, Lu, & Davis, 2002)

A two-way ANOVA was conducted to analyze the main effects of drug treatment (losartan or saline) and time (Pre-FPS, Post-FPS 1, Post-FPS 2). A Paired Sample T-test was performed to analyze whether extinction occurred between Post-FPS 1 and Post-FPS 2.

Experiment 2

Subjects

All mice (N = 16) in the experiment were 3-4 month old wild type C57BL/6J males from Jackson Laboratory (Bar Harbor, ME). All procedures were approved by the Institutional Animal Care and Use Committee of George Washington University and were in compliance with the National Institutes of Health guidelines.

Drugs

Losartan (Sigma-Aldrich, catalog no. 61188) was administered intraperitoneally (i.p.) at a dose of 10 mg/kg in 0.2 ml of a vehicle of 0.9% isotonic sterile saline. Saline was administered to the control group. The mice received a single dose of losartan or saline 40 minutes prior to Fear-Potentiated Startle Test 1.

Habituation and Fear-Potentiated Startle Pretest

For two days, the mice were exposed and acclimated to a non-restrictive clear acrylic cylinder (SR-Lab startle response system, San Diego Instruments) attached to an accelerometer that measured mV reactivity. On the first day, subjects were exposed to a 17 min baseline reactivity test which consisted of 48 0.5 sec white noise bursts at varying decibel levels: 65, 81, 90, 95, 100, 105, 110, and 115 dB and a randomized inter-trial interval (between 0-30 sec). This information was used to select a dB level (100 dB) that evoked a recognizable startle response without causing a ceiling effect.

On the second day, the animals were received a pre-FPS test to measure baseline potentiation to the tone conditioning since fear reactivity in mice varies greatly within groups (Falls, 2002). The FPS test consisted of 5 min silence and 10 “leader trials” (0.5 sec 100 dB noise burst) to acclimate the mice to the chamber and stabilize reactivity. This was followed by 12 tone (30 sec, 12 kHz, 60 dB) trials that co-terminated with a white noise burst (0.5 sec, 100 dB) and were interspersed with 12 noise alone trials (0.5 sec, 100 dB), and 12 no-stimulation trials (30 sec silence). No-stimulation trials were used to measure baseline movement within the cylinders. Each trial was followed by a 30 sec inter-trial interval.

Fear Conditioning

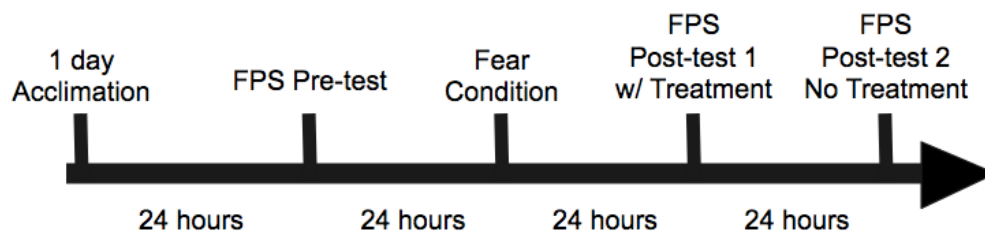
Auditory fear conditioning was performed using the SR-Lab startle response system (San Diego Instruments). A metal shock grid was placed in the non-restrictive clear acrylic cylinders for the purpose of fear conditioning. Additionally, the environment was modified to reduce contextual conditioning (ammonia cleaner was used instead of alcohol cleaner and colored paper was put around the walls). Animals were habituated to the chambers in two 20 min sessions two days prior to fear conditioning protocol and both the saline and losartan groups received to the fear

conditioning protocol. The protocol consisted of 5 min habituation period followed by 5 CS-US pairings: a 30 sec auditory tone (6 kHz, 75 dB) co-terminating with a mild footshock (0.5sec 0.5 mA). There was a 3 min 30 sec inter-trial interval between each pairing. The conditioning chambers were cleaned with ammonia cleaner between each trial and the animals were returned to their home cages for 24 hours following conditioning.

Fear-Potentiated Startle Extinction Training

The FPS extinction test was performed using the SR-Lab Startle Response System (San Diego Instruments). The FPS protocol used in the pretest was also used in both posttests. Mice were injected with losartan or saline 40 min prior to the first FPS test and returned to their home cages. The conditioning chambers were cleaned with 70% ethanol between each test and the animals were returned to their home cages for 24 hours following FPS extinction training. The following day, the mice were not injected with losartan or saline and received the second FPS test.

Timeline of Behavioral Assessment for Experiment 2



Analysis

To analyze fear conditioning in mice in the startle apparatus, a baseline measure of the accelerometer was taken. The accelerometer was found to have a baseline measure of 7 mV and this information was used to calculate freezing in animals. Freezing was assumed if animals presented maximal movement of 7 mV. Percent Freezing was calculated for each time point by

averaging movement and dividing by the number of trials for each inter-trial interval (time between tones). Fear conditioning was then assessed by comparing freezing behavior prior to the initial shock (Pre-US freezing) to freezing behavior following the initial shock (Post-US freezing). A two-way ANOVA with time and drug as factors was used to confirm conditioning and similarity between treatment groups.

Again, Fear Conditioning was assumed based on a significant increase in FPS from Pretest to Posttest1 (Falls, 2002). It was assumed that animals returned to baseline and had extinguished to the fear cue if there was no significant difference between Pretest and Posttest 2. This assumption was based on the data that showed that animals who had received extinction treatment in other experiments no longer exhibited FPS when exposed to the CS prior to a startle stimulus (Walker, Ressler, Lu, & Davis, 2002).

Mirroring analysis for Experiment 1, fear-potentiated startle was calculated based on a ratio comparing tone + noise trials to noise alone trials using the following formula: Percent fear-potentiated startle (%FPS) = $[(\text{tone} + \text{noise burst} - \text{noise burst alone}) / \text{noise burst alone}] \times 100$. To determine fear potentiation, the average reactivity in mV was calculated for each animal for each set of trials: [No-stimulation], [noise burst alone], and [tone + noise]. Percent FPS was then calculated using the above formula for each animal.

A two-way ANOVA was conducted to analyze the main effects of drug treatment (losartan or saline) and time (Pre-FPS, Post-FPS 1, Post-FPS 2). A Paired Sample T-test was performed to analyze whether extinction occurred between Post-FPS 1 and Post-FPS 2.

Results

Study 1

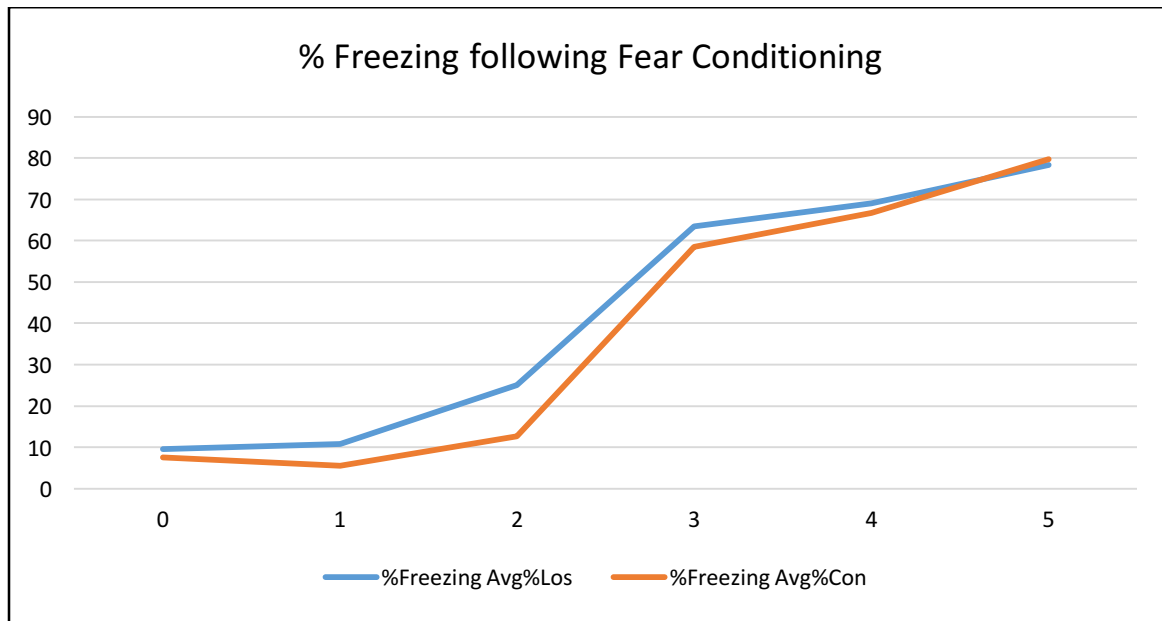


Figure 1. A significant increase in %Freezing from time-point 1 to time-point 5 is used to show that fear conditioning occurred over time. Time-point 0 refers to the 5-minute habituation period prior to CS-US conditioning. Each of the following time points indicates the Average %Freezing over the course of the 30 second tone (CS).

Fear Conditioning. Testing for fear acquisition was conducted prior to drug exposure and animals exhibited normal levels of fear conditioning within the Coulbourn Fear Conditioning apparatus. This was measured based on an increase in %freezing to the conditioned stimulus (tone) over the course of a 5CS-US test (Figure 1). A two-way ANOVA with drug (losartan or saline) and time (pre-US exposure vs post-US exposure) was conducted. As expected, there was no significance of drug indicating that there was no difference between groups prior to drug treatment. There was a significant main effect of time, $F(1,6)=75.729$, $p < .001$, indicating that all mice were fear-conditioned to the tone.

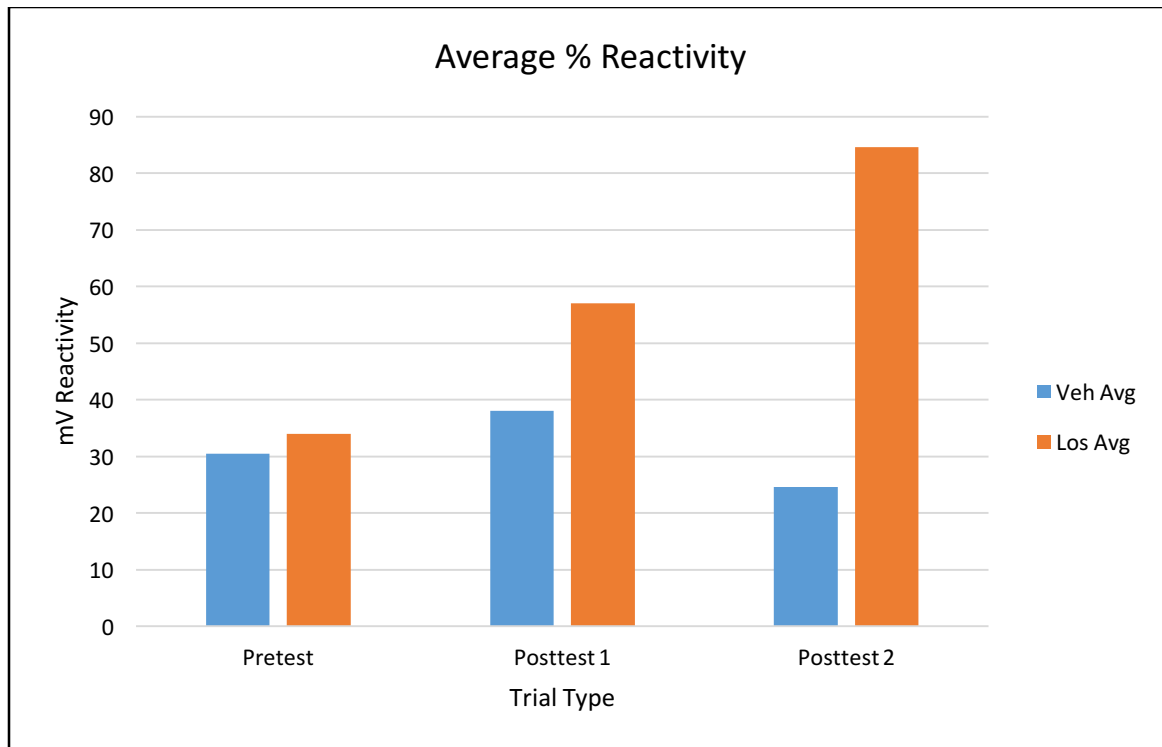


Figure 2. While this figure does not show significance, it was used to show trends between groups treated with losartan vs. saline.

Fear-Potentiated Startle. A two-way ANOVA, including drug (losartan or saline) and time (pretest, posttest 1, posttest 2) as factors, was conducted. For these pilot data, there were no significant main effects and the interaction was not significant. However, there was a trend for a main effect of time, $F(2,10) = 2.869$, $p = .104$. This trend reflects higher reactivity during posttest 1 and posttest 2 compared with pretest (Figure 2).

Since there was a trend for an effect of time, post-hoc paired sample T-tests were performed to assess whether FPS was exhibited in a specific group. There was no significant difference within group comparisons for the saline group. While there was no significant difference within group comparisons for the losartan group, there was a trend towards significance from pretest to posttest 2.

Study 2

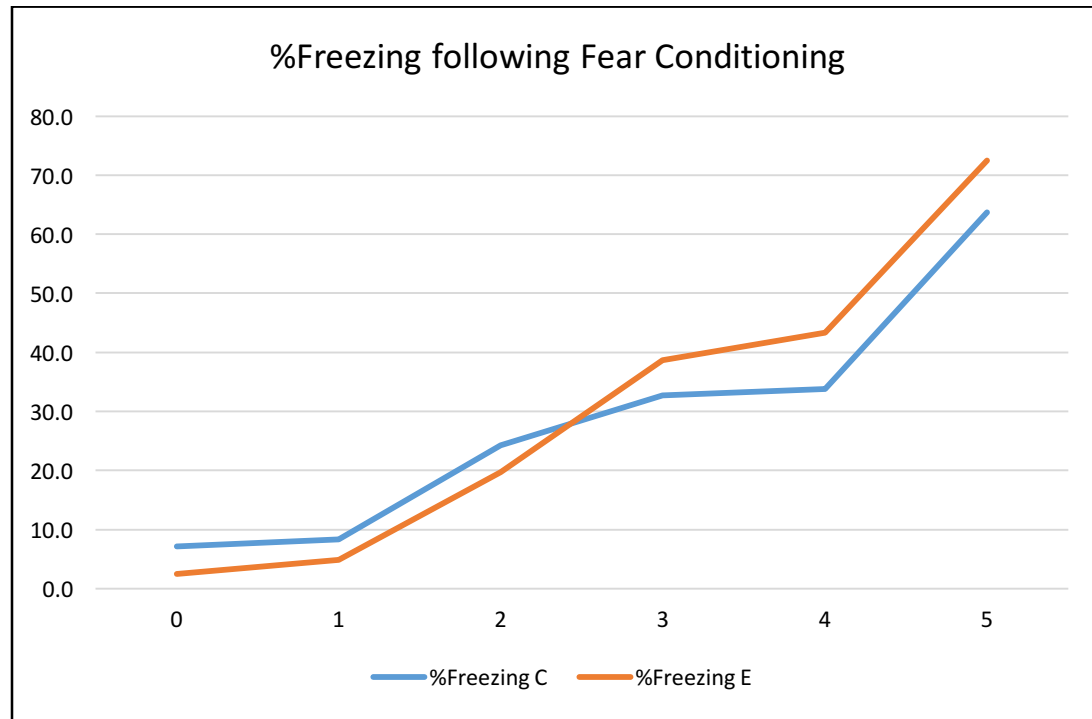


Figure 3. This figure shows the average increase in % Freezing over time. Fear was acquired over the course of 5 tone-shock (CS-US) pairings and freezing was measured over the course of each 30 second tone, prior to the fear stimulus.

Fear Conditioning. In this experiment, testing for fear conditioning was conducted prior to drug exposure and animals exhibited normal levels of fear conditioning within the San Diego Labs Fear Conditioning apparatus (Figure 3). Conditioning was assessed based on a significant increase in %freezing to the conditioned stimulus (tone) over the course of a 5CS-US test. A two-way ANOVA with drug (losartan or saline) and time (pre-US exposure vs post-US exposure) was conducted. Again, there was no significance of drug interaction. There was a significant main effect of time, $F(1,14)=122.300$, $p < .001$, indicating that all mice were fear-conditioned to the tone.

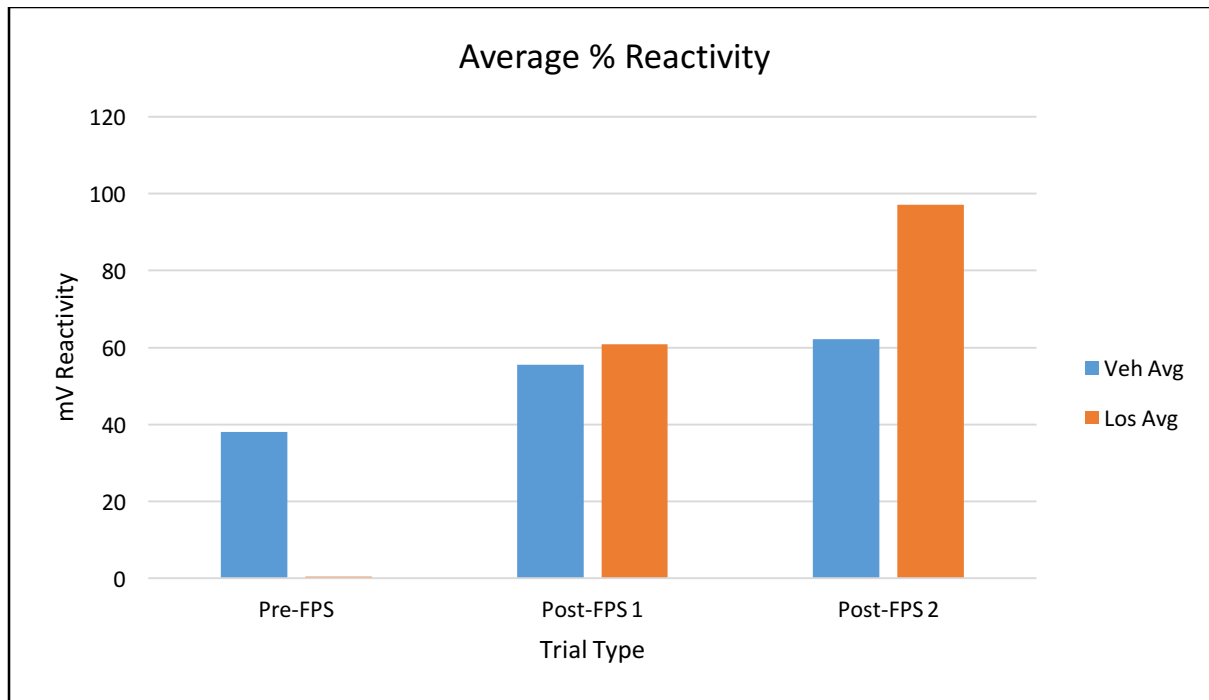


Figure 4. The figure shows a significant increase in FPS Response between time point 1 (Pretest) to time point 2 (Posttest 1) for mice treated with losartan.

Fear-Potentiated Startle. A two-way ANOVA, including drug (losartan or saline) and time (pretest, posttest 1, posttest 2) as factors, was conducted. For these data, there were a significant main effect of time, $F(2,28) = 9.346$, $p = .001$. This trend reflects higher reactivity during posttest 1 and posttest 2 in comparison with the pretest (Figure 4). There was no main effect of drug or of interaction, but there was a trend for interaction (drug*time) ($F(2,28)=3.319$, $p = .051$).

Since fear-potentiated startle is assessed based on a significant change in potentiated startle reactivity between pretest and posttest and since there was a trend for drug*time interaction, post-hoc tests were performed. Data were split based on saline or losartan treatment and a Paired Sample T-Test was performed. For saline treatment, there was no significant difference from pretest ($M=38.14$, $SD=33.07$) and posttest 1 ($M=55.5$, $SD=49.60$); $t(7) = -1.269$, $p=.245$. There was also no significant difference from pretest ($M=38.14$, $SD=33.07$) to posttest 2 ($M=62.20$,

SD=46.64); $t(7) = -1.051$, $p=.328$. However, for losartan treatment, there was a significant difference between pretest ($M=.5364$, $SD=27.84$) to posttest 1 ($M=60.87$, $SD=32.94$); $t(7) = -7.452$, $p<.001$, as well as between pretest ($M=.5364$, $SD=27.84$) to posttest 2 ($M=97.01$, $SD=39.63$); $t(7) = -5.441$, $p = .001$. These results indicate that animals expressed fear-potentiated startle following conditioning and further that drug treatment combined with FPS extinction training did not result in a return to baseline startle reactivity.

Discussion

There were two principal goals for this study. The first was to examine whether the FPS paradigm resulted in extinction learning, albeit delayed in comparison to the typical extinction paradigm. The second goal was to examine whether fear conditioned animals that were treated with losartan would exhibit increased retention of extinction learning in comparison to an untreated group, in spite of the slightly aversive startle stimulus associated with the CS (tone). The idea for this study was derived from human studies that suggest that individuals with PTSD may frequently find themselves in environments of heightened stress as well as animal studies that have shown that the fear-potentiated startle paradigm may be an extinction paradigm that interrupts effective CS processing (Morgan III et al., 1995; DSM V, 2013; Walker et al., 2002). Based on prior knowledge of the renin-angiotensin system and the interactions of losartan with the AT1 and AT2 receptors, it was hypothesized that treatment with losartan would enhance extinction learning even in the presence of the startle stimulus associated with the FPS paradigm (Marvar et al., 2014; Unger et al., 2015; Khoury et al., 2012; Marinzalda, Pérez, Gargiulo, Casarsa, Bregonzio, & Baiardi, 2014). (Falls, 2002)

While overall, the scope of these data were too limited to provide conclusive evidence for either hypothesis, both experiments produced some interesting findings. Interestingly,

counter to the hypothesis concerning losartan, the results from Experiment 1 showed that the reactivity to the startle stimulus increased from day 1 to day 2 in mice treated with losartan. In comparison, there was not even a trend for significance in change over time for mice who were fear conditioned and treated with saline.

There were a number of limitations to the interpretation of Experiment 1. To start, the data from this study was overall insignificant. This was largely attributed to the small sample size. Fear-potentiated startle behavior is extremely variable in mice, and as a result, a larger sample size was necessary to validate any trends exhibited in Experiment 1.

Experiment 1 was thus used to validate a secondary replication of the study. The trends from these data were perceived as implying that losartan might have an effect of increasing fear expression in the mice who received extinction training using the FPS paradigm. The data also implied that FPS could be used as an extinction study. This was indicated by the insignificant but slight decrease in percent-potentiation exhibited by saline treated mice from posttest 1-posttest 2 in Figure 2.

Experiment 2 mirrored Experiment 1 with a few significant changes. First, Experiment 2 had a larger group size of eight mice per group. Additionally, the tone was changed from 6-kHz to 12-kHz based on information that a 12-kHz tone may induce less potentiated fear than the 6-kHz tone prior to fear conditioning (Falls, 2002). This change was made based on a consideration that the startle reactivity of the mice in the pretest in Experiment 1 might have exhibited potentiation to the tone prior to fear conditioning resulting in a ceiling effect. Lastly, Experiment 2 was shortened since only one hearing test was deemed necessary and since fear conditioning was conducted in the startle apparatus.

In spite of the changes, Experiment 2 produced similar results exhibited in Experiment 1. Importantly, the FPS exhibited by the losartan group between pretest to posttest increased significantly. This was expected since a significant increase from pretest to posttest indicated that fear conditioning had occurred. Unexpectedly, however, the FPS exhibited by the losartan group from pretest to posttest 2 also remained significant. This indicated the extinction learning had not occurred.

Interestingly, the saline group did not show a significant increase in potentiated startle from pretest to posttest 1 and further did not show a significant increase from pretest to posttest 2. Each fear conditioning session was conducted with mixed groups, so fear conditioning of all mice was confirmed. In an effort to confirm that fear potentiation was occurring, data for the saline treated animals in Experiment 2 were combined with data from mice in preliminary experiments that had been treated to the same protocol up to the implementation of posttest 1. The new subject population of 14 mice produced a significant difference between pretest and posttest 1 indicating that a larger sample size would have been helpful for this experiment.

The small sample size created additional caveats for this study and the results are thus inconclusive. To begin, this study could not be used to prove the FPS paradigm was an adequate fear extinction paradigm. In order to assess whether the subjects were extinguished to the fear cue, it would have been necessary for either the saline or losartan group to show significant potentiation within the initial posttest and then return to baseline reactivity levels. Since the saline group did not exhibit a significant increase in fear-potentiated startle at any time point, neither potentiation or extinction to the CS could be confirmed. Since the losartan group showed fear potentiation in both pretest to posttest 1 and in pretest to posttest 2, only a failure to extinguish could be confirmed.

In spite of the limitations, these data could still provide some insights for future studies. While this FPS paradigm could not be confirmed as an effective method of extinction, it is possible that the methods used in this experiment simply needed to be adjusted. For example, it may be helpful in future studies to utilize a longer FPS extinction period. This study provided the animals with only 15 “extinction” (tone + startle) trials per test. Many classic extinction tests are significantly longer, exposing the animals to 30+ tones to ensure full extinction to the CS (Marvar et al., 2014; Walker et al., 2002). Further study may find that the FPS paradigm could be used as a successful extinction paradigm if further adjustments are made.

Alternatively, it is possible that the FPS paradigm used was a successful extinction paradigm for the saline group and that the effects of extinction would have been more apparent with a larger sample size. Further, the group treated with losartan showed significant potentiation on both days and in both experiments even with the small sample sizes. Since the increased potentiation was consistent between experiments, these results may be an interesting target for future study. It is possible that the startle noise interfered with CS processing and as a result, losartan, like d-Cycloserine augmented the fear memory (Reinecke et al., 2015). Following this line of thought, this study could provide a few implications concerning losartan’s effect on memory. The study published by Marvar et al. (2014) stated that losartan might improve extinction learning by improving consolidation of the new memory, but consolidation is the initial storage of a new memory. The assumption made by Marvar et al. (2014) suggests that losartan may improve consolidation of a new extinction memory that then replaces or overpowers the old fear memory.

Though this study provided inconclusive results, subjects treated to losartan consistently demonstrated an increase in potentiation to the tone following from posttest 1 to

posttest 2, something that did not occur with the saline group. The startle stimulus is not harmful to the mice, and so if losartan augments consolidation of extinction memory, it would be counterintuitive for the new memory to result in a higher level of potentiated startle as was seen in this experiment.

Though it should be judged carefully, an alternative suggestion is that losartan might augment *reconsolidation* of memory. Reconsolidation occurs when a memory is retrieved and then returned to long term memory (Duvarci & Nader, 2004). It has been found that when a memory is retrieved, it reenters a labile state and can be altered or come to be associated with other memories (Sierra, et al., 2013; Duvarci & Nader, 2004). It is possible that treatment with losartan resulted in reconsolidation of the trauma memory associated with a new memory of the general discomfort induced by the startle noise. That said, to assess losartan's effects on learning and memory based on the data in this study requires broad assumptions.

In order to draw conclusions about the FPS paradigm as a true extinction model, future research would require a larger sample population, as well as a reevaluation for the FPS extinction method. For example, aside from elongating animal exposure to the FPS paradigm, it might be more effective to have multiple exposure sessions to the paradigm instead of only two as was performed in this study. Further, if the FPS paradigm is to be used as a model of the imperfect nature of human psychotherapeutic treatment, it may be helpful to include segments within the paradigm where the animal is exposed to the fear cue without an accompanying startle stimulus, thus modeling successful therapeutic sessions.

A number of reevaluations and further tests would be necessary to draw conclusions about losartan in relation to the FPS paradigm. It is important to note that the possibility that

losartan may potentiate a stress reaction is counter to much of the known information pertaining to the drug.

For one, losartan is a common anti-hypertension medication and is not associated with side effects of increased levels of stress or anxiety. Additionally, the overarching basis for this study ties back to the Khoury 2012 study which found that overall, patients exhibited fewer symptoms of distress when taking losartan as a medication. Further, acknowledging that many patients take hypertension medications such as losartan on a regular basis, the study conducted by Marvar et al. 2014 found that continuous injection of losartan in mice did not impact fear learning, but still improved extinction retention following traditional extinction training.

In conclusion, further analysis will be necessary to establish whether the FPS paradigm can act as a true method for modeling the exposure therapy within the lab. Lastly, regardless of the fact that the results were inconclusive, it is undeniable that the group treated with losartan exhibited significantly higher levels of fear potentiation in comparison to the saline group. It is thus still relevant and necessary to further examine the actions of the ARB and further assess whether losartan may be a worthwhile medication for the purpose of augmenting learning retention.

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